Expression of resistance markers to methotrexate predicts clinical improvement in patients with rheumatoid arthritis

J Wolf, T Stranzl, M Filipits, G Pohl, R Pirker, B Leeb, J S Smolen

Background: Methotrexate is transported into the cell by the reduced folate carrier (RFC) and out of the cell by members of the multidrug resistance protein family (MRP). Transport proteins may affect the therapeutic efficacy of this drug in patients with rheumatoid arthritis.

Objective: To investigate the potential benefit of the presence of RFC and the absence of functional MRP for the efficacy of methotrexate treatment.

Methods: The study involved 163 patients (116 female, 47 male; mean age 59.5 years) on methotrexate (mean weekly dose 12.2 mg). RFC was determined using reverse transcriptase polymerase chain reaction, and MRP function by flow cytometry, using a calcein acetoxymethylesther/probenecid assay. Clinical response to methotrexate was evaluated by the EULAR response criteria and the ACR 20% improvement criteria. The clinical data were obtained at the beginning of methotrexate treatment and at the time of blood sampling during ongoing therapy. Patients were divided into four groups according to the presence (+) or absence (−) of RFC and functional (f) MRP.

Results: IMRP+/RFC− and fMRP−/RFC− patients more often had good EULAR response rates (60%, p = 0.014, and 53%, p = 0.035, respectively) in comparison with the fMRP+/RFC+ group (29%); IMRP+/RFC− patients had a low frequency of good disease activity responses.

Conclusions: Absence of IMRP plus presence of RFC did not prove to be related to beneficial effects of methotrexate, but the lack or the presence of both fMRP and RFC led to a significantly better therapeutic outcome. Determination of these markers may predict responsiveness to methotrexate.
All clinical and serological variables were evaluated prospectively in the course of routine clinical care, with blinded to the results of RFC and fMRP testing. This evaluation included joint counts, patient and physician global assessment and patient pain assessment by visual analogue scale (VAS), and acute phase reactants. Therapeutic response was evaluated using the EULAR response criteria, which employ the 28 joint disease activity score (DAS28). The DAS was determined prospectively at the initiation of methotrexate treatment and at the time of the present investigation. A DAS28 of more than 5.1 indicates high disease activity; between 3.2 and 5.1, moderate disease activity; below 3.2, low disease activity. A decrease in DAS28 of >1.2 represents a good EULAR response (unless DAS is in the high disease activity range at the end of observation); a decrease of >0.6 but <1.2 is considered a moderate response; a change of <0.6 (or high disease activity whatever the change in the DAS) is regarded as lack of clinical response. The ACR 20% (ACR20) was also evaluated—that is, a 20% decrease in the number of swollen and painful joints and in at least three of the following: patient pain assessment, patient and physician global assessment, erythrocyte sedimentation rate (ESR) or C reactive protein, and health assessment questionnaire (HAQ) score.

**Determination of RFC mRNA expression and MRP function**

The role of the individual resistance factors and their method of determination are shown in table 1. RFC mRNA expression was determined using reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation. Total RNA was isolated using an RNA isolation kit (RNEasy; Qiagen, Valencia, California, USA). cDNA was obtained using Superscript II reverse transcriptase (Life Technologies, Rockville, Maryland, USA). RT-PCR was then carried out. Thirty five PCR cycles were completed (denaturation, annealing, polymerisation), followed by a final elongation step. For RFC, forward primer RFC-617 (5'-CCAAGCGCA GCCTTTCTTTCAACC-3', bases 617–640) and reverse primer RFC-949 (5'-CCAGCAGCGTGAGGCACGACATGCGC-3', bases 924–949) were used, leading to a product of 333 base pairs (bp). RT-PCR products were separated by 2% agarose gel electrophoresis with 0.5 μg/ml ethidium bromide for DNA visualisation.

We used β2 microglobulin as a housekeeping gene. RFC+ samples were defined by visual detectability of RFC-PCR products after electrophoresis; RFC− samples yielded no visible bands of PCR products (low RFC mRNA copy number). As there was a large variation in expression of RFC (from not visible to very intense bands) and as two distinct patterns were found (negative or positive), further densitometric analyses of the bands was not undertaken. Human erythroleukaemia K562 cells were included as a positive control in all assays.

MRP function was measured by flow cytometry of total mononuclear blood cells using a calcein acetoxyethylster/probenecid assay with a final probenecid concentration of 1 mM. In brief, cells were preincubated with warm RPMI 1640 medium after several washing steps to remove any remaining serum components, followed by a 15 minute incubation with calcein acetoxyethylster. After 90 minutes' incubation in medium with or without probenecid as modulator of MRP activity, fluorescence was measured with a FACScan cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). fMRP was defined as the transport of methotrexate and its successful blocking by probenecid: fMRP+ means transport of methotrexate through the cell membrane, inhibitable by probenecid; fMRP− means no efflux because of the absence of any transport mechanism, or transport of methotrexate by another mechanism not inhibitable by probenecid, such as P-glycoprotein (in both cases there is no functional MRP present).

The mean fluorescence index for fMRP+ was 1.12 (95% confidence interval (CI), 1.07 to 1.32), and for fMRP− 0.93 (0.9 to 0.97). The cut off level for MRP functionality was defined as >1.00.

**Table 1** Resistance markers for methotrexate

<table>
<thead>
<tr>
<th>Resistance marker</th>
<th>Location</th>
<th>Function</th>
<th>Method of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced folate carrier (RFC)</td>
<td>Cell membrane protein</td>
<td>Influx of reduced folate and methotrexate</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Multidrug resistance proteins (MRP 1–4)</td>
<td>Cell membrane proteins</td>
<td>Efflux of methotrexate, cellular detoxification</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Folatepolyglutamyl synthetase (FPGS)</td>
<td>Cytoplasmic enzyme</td>
<td>Polyglutamylation of methotrexate and folate (presumed increase in efficacy)</td>
<td>RT-PCR (not done)</td>
</tr>
</tbody>
</table>

RT-PCR, reverse transcriptase polymerase chain reaction.

**Table 2** Mean dose of methotrexate and other disease modifying antirheumatic drugs

<table>
<thead>
<tr>
<th>Methotrexate dose*</th>
<th>Folic acid suppl</th>
<th>Chloroquine</th>
<th>Sulfasalazine</th>
<th>Ciclosporin</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.1 (3.9) mg</td>
<td>29 (41%)</td>
<td>11 (15.7%)</td>
<td>1 (1.4%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>12.1 (3.7) mg</td>
<td>9 (28%)</td>
<td>3 (9.3%)</td>
<td>1 (3.1%)</td>
<td>1 (3.1%)</td>
</tr>
<tr>
<td>12.7 (3.7) mg</td>
<td>12 (36%)</td>
<td>1 (3.0%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12.0 (3.9) mg</td>
<td>9 (32%)</td>
<td>4 (14.2%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are n (%) or mean (SD).

fMRP, functional multidrug resistance proteins; RFC, reduced folate carrier; suppl, supplementation; +, positive; −, negative.
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n = 32
n = 28
n = 33

n = 70
n = 32
n = 33
n = 28

**Figure 1** Good, moderate, and low DAS28 response in each of the four groups. Probability (p) values relate to good responses in each group compared with fMRP_+/RFC_+ patients. Mean change in DAS was significantly lower among fMRP_−/RFC+_ patients than among either fMRP_−/RFC_− or fMRP_+/RFC_+ patients. DAS28, 28 joint disease activity score; fMRP, functional multidrug resistance proteins; RFC, reduced folate carrier.

**Table 3** Mean 28 joint disease activity score (DAS28) and reduction in DAS28

| Groups according to resistance markers | Change in DAS28 at start of treatment | Mean DAS28 at time of blood sampling p Value v \( \text{fMRP}^{-}/\text{RFC}^{+} \) | fMRP, functional multidrug resistance proteins; RFC, reduced folate carrier.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>fMRP_−/RFC_− (n = 70)</td>
<td>0.71 (1.27)</td>
<td>3.08 (1.02)</td>
</tr>
<tr>
<td>fMRP_−/RFC_+ (n = 32)</td>
<td>1.23 (1.44)</td>
<td>3.22 (1.30)</td>
</tr>
<tr>
<td>fMRP_+/RFC_− (n = 33)</td>
<td>3.12 (1.36)</td>
<td>3.38 (1.10)</td>
</tr>
<tr>
<td>fMRP_+/RFC_+ (n = 28)</td>
<td>1.09 (1.29)</td>
<td>3.39 (0.95)</td>
</tr>
</tbody>
</table>

Values are mean (SD).
The observation that methotrexate had only weak efficacy in fMRP−/RFC+ patients may not necessarily indicate that the influx of methotrexate into the cell (as mediated by RFC) is insufficient to allow methotrexate to exert its effect as a DMARD within the cell. Other pathways for ingress of methotrexate into the cell which were not examined in this study might be more efficient in this respect.17 In addition, the influence of MRP on methotrexate may be more complex than simply transporting the drug out of the cell. Moreover, given the result in this subset of fMRP−/RFC+ patients, it is unlikely that methotrexate upregulates MRPs, as has been described for other agents such as cisplatinum compounds or the glucocorticoid dexamethasone.11,12 et al have reported that in drug-depleted breast cancer cells, exposure to low-dose methotrexate under certain conditions results in a decrease in RFC-1 expression, and the initial rate of methotrexate uptake over time decreased to 22% of the baseline value.14 As the current investigation was carried out 12 weeks after the initiation of methotrexate, such effects, if present in vivo, could also be responsible for some of our observations.

Among rheumatoid patients treated with methotrexate, 30–50% do not fulfill response criteria. These data, obtained in clinical trials, are confirmed here in a cohort of patients followed during routine clinical care. However, the highest frequency of non-responders (57%) was observed among the fMRP−/RFC+ patient population, while the lowest frequency of DAS28 non-responders (37%) was seen among fMRP−/RFC− patients. The results we obtained also suggest an impact on clinical decision making because they could provide rheumatologists with a tool to predict responsiveness to methotrexate. As 57% of the fMRP+RFC+ and fMRP−RFC− patients had a good clinical response, compared with only half that proportion in fMRP−/RFC+ patients, determination of these proteins may be helpful in predicting the probability of responsiveness to methotrexate.

At present, no responsiveness to traditional DMARDS nor responsiveness to biological agents can be predicted on clinical or laboratory grounds. We have revealed a potential tool for discrimination of likely methotrexate responders from likely non-responders. Further studies in other patient populations will be needed to confirm these results. However, once confirmed, this finding could be a major breakthrough in predicting responsiveness to treatment in rheumatoid arthritis.

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